Integration of genetic pathways regulating cellular stress response in *Caenorhabditis elegans*

PhD thesis

Janos Barna

Doctoral School of Biology, Eötvös Loránd University
Head of Doctoral School: Anna Erdei DSc, member of HAS

Doctoral Program in Classical and Molecular Genetics
Head of Doctoral Program: László Orosz DSc, member of HAS

Supervisor: Krisztina Vellai-Takács PhD assistant professor
Consultant: Tibor Vellai DSc associate professor

Eötvös Loránd University, Faculty of Science, Institute of Biology
Department of Genetics

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Introduction

Environmental stress induces distinct cell protective mechanisms such as autophagy and heat shock response to maintain cellular homeostasis. Autophagy eliminates damaged cellular components while heat shock response ensures the structural integrity of cytosolic proteins. During heat shock response molecular chaperones help to refold or degrade damaged proteins, thereby preventing the formation of protein aggregates.

The insulin/IGF-1 (*insulin-like growth factor-1*) and TGF-β (*transforming growth factor-beta*) signaling pathways control metabolism, aging, stress response and development in the nematode *Caenorhabditis elegans* [1-5]. Activity of these pathways is decreased upon harsh environmental conditions, such as starvation, high population density and high temperatures. This response leads to lipid accumulation in the adipose tissues, lifespan extension, an enhanced tolerance against heat stress, and a switch in the developmental program from normal reproductive growth to dauer development [6, 7]. The membrane bound receptor guanylate cyclase (GC) DAF-11 is an upstream regulator of both pathways. It generates the messenger molecule cGMP, which in turn activates the TGF-β ligand DAF-7 and the insulin/IGF-1 ligand DAF-28 [8, 9].

The insulin/IGF, TGF-β and cGMP pathways are implicated in the synthesis of dafachronic acid (DA), a steroid hormone required for reproductive development. A key step in DA synthesis is catalyzed by DAF-9/cytochrome P450. The expression of *daf-9* gene is regulated by DAF-7/TGF-β and insulin/IGF-1 signaling [6, 10].

Mutations blocking insulin/IGF-1 or TGF-β signaling promote dauer development in a temperature-dependent fashion: penetrance of the dauer constitutive Daf-c phenotype in *daf-2* and *daf-7* mutants correlates with the ambient temperature. Besides temperature, nutritional status and population density also affects dauer larva formation in mutants defective for TGF-β or insulin/IGF-1 signaling.

In eukaryotes, the transcription factor HSF-1 protects cells against protein damaging stress through initiating a conserved transcriptional program [11-13]. Upon temperature increase, HSF-1 becomes activated via trimerization and phosphorylation, then translocates into the nucleus to promote the transcription of genes that encode heat shock proteins (HSPs), also called molecular chaperons [13]. These factors largely contribute to the protection of cells from protein-damaging stress. Besides its role in stress response HSF-1 has also been shown to influence aging and development [11, 14]. In *C. elegans*, insulin/IGF-1 signaling inhibits

HSF-1 activity [14]. When insulin/IGF-1 signaling is attenuated, HSF-1 and the insulin/IGF-1 signaling target DAF-16/FoXO (Forkhead box-O) stimulate the expression of genes required for longevity and stress resistance [1].

Aims of the study

Protein damage caused by various environmental stresses induces distinct cell protective mechanisms, such as autophagy and heat shock response. The aim of my work was to discover signaling crosstalk between genetic pathways that regulate cellular stress response in *C. elegans*.

Most target genes of HSF1 encode heat shock proteins. However, it has been shown that besides activating expression of molecular chaperone genes, HSF1 also regulates the transcription of genes involved in cell differentiation and development. Our aim was to determine new direct targets of HSF-1, which regulate cell differentiation and development in *C. elegans* using *in silico* methods.

The HSF-1 consensus binding site (TTCNNGAANNTTC) is well defined and conserved in eukaryotes. Conservation of a DNA sequence suggests conservation in functionality. Thus, we planned to find putative HSF-1 target genes by testing the conservation of the consensus HSF-1 binding site in the 5' regulatory region of orthologous genes in different *Caenorhabditis* species. Among these genes we wished to further analyze those ones that are implicated in the regulation of animal development or identified as components of signaling pathways regulating cellular stress response.

The transcription factor HSF-1 is activated upon increase of environmental temperature. Therefore, we wished to monitor the expression of putative target genes at different temperatures, in *hsf-1* loss of function and HSF-1 overexpressing mutant backgrounds, using quantitative real time PCR.

We planned to analyze *in vivo* the expression of putative HSF-1 target genes in various mutant backgrounds using gfp-labeled reporter constructs. To prove that HSF-1 directly regulates the transcription of a given target gene, we intended to create reporter constructs containing the wild-type or mutated version of the putative HSF-1 binding sites.

We wished to identify the regulatory relationship between HSF-1 and its putative target genes by epistasis analysis.

Identifying HSF-1 paralogs

In vertebrates heat shock transcription factors constitute a multiprotein family. However, in invertebrates only one single heat shock transcription factor has been identified so far. Our aim was to perform an *in silico* search in the *C. elegans* genome for genes encoding heat shock transcription factor-like proteins and perform their genetic analysis.

Regulation of autophagy by TGF-β signaling

Autophagy, a cellular self-degradation process of eukaryotic cells, was studied thoroughly in our laboratory. Based on our preliminary findings, we assumed that TGF-β signaling influences cell growth via regulating autophagy. To support this hypothesis we wanted to monitor autophagic activity *in vivo* in hypodermal seam cells of various mutant strains deficient in TGF-β signaling, using a *gfp::lgg-1* reporter that labels autophagosomal structures.

Methods

Identifying HSF-1 binding sites *in silico* in the *C. elegans* genome

Wormenhancer (*Open genomics*) and cisRED softwares were used to identify putative HSF-1 binding sites in the *C. elegans* genome. Since evolutionary conservation of an element implies a conserved functionality, only those sites were selected for further analysis whose position and sequence were conserved in the genome of two closely related *Caenorhabditis* species, *C. briggsae* and *C. remanei.*

In vivo expression analysis

To analyze the expression of genes of interest we generated GFP reporter constructs using standard recombinant DNA techniques. *In vitro* mutagenesis was performed to make reporter constructs containing a mutant version of a given HSF-1 binding element in order to explore the functionality of the putative binding site. Transgenic animals were generated using biolistic transformation. Expression analysis was performed by fluorescent microscopy.

Analysis of *daf-7* expression by quantitative real time PCR

Expression of *daf-7* (a putative HSF-1 target gene) was also analyzed by qRT-PCR. Samples were extracted from 100–200 synchronized L1 larvae, using a PureLink™ Micro-to-Midi Total RNA Purification System (*Invitrogene*). RNase-free DNase I (*Fermentas*) was used to eliminate genomic DNA contamination. DNA-free RNA samples were then converted to cDNA, using random hexamers by RevertAid First Strand cDNA Synthesis Kit.

Amplification reactions were carried out in a total volume of 20 µl, using LightCycler® FastStart DNA Master SYBR Green I. The amplification was performed using LightCycler® 2.0 Instrument (Roche). Relative gene expression data were calculated by comparative CT method using $2^{-\Delta\Delta CT}$ formula.

Lifespan assays

Life span assays were carried out at 25°C. Synchronized L4/young adults were transferred to NGM plates supplemented with FUDR (5-fluoro-2'-deoxyuridine). We determined the number of dead animals each day during the experiment. Animals were considered dead when they stopped pharyngeal pumping and responding to touching.

Dauer assay

A synchronous population of animals were grown at the appropriate temperatures of the assay (20°C, 23°C, 26,5°C), and percentage of dauer larvae was scored when animals have reached the L4 larval stage, typically after 72, 60 and 44 hours, respectively.

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Results

The HSF-1 transcriptionfactor represses *daf-7*

- We have identified a conserved HSF-1 binding site in the 5' regulatory region of *daf-7*, using *in silico* methods.
- Using quantitative RT-PCR, we showed that at elevated temperatures *daf-7* mRNA levels are decreased in an HSF-1 dependent manner.
- Epistasis analysis showed that *hsf-1(sy441)* hipomorphic mutation suppresses the dauer constitutive phenotype of *daf-1l(m47)* and a *daf-21(p673)* mutants at 20 and 23°C.
- *In vivo* expression analysis of a *daf-7::gfp* reporter (*ksIs2*) in ASI neurons showed that HSF-1 function is required for the repression of *daf-7* in animals defective for DAF-11/GC and DAF-21/Hsp90: *daf-11(-); hsf-1(sy441)* and *daf-21(p673); hsf-1(sy441)* double mutant animals displayed wild-type levels of *daf-7* expression. A similar result was observed in *daf-11(-)* mutant animals when *hsf-1* was inactivated either by a null mutation or by RNAi.
- To prove direct transcriptional regulation of *daf-7* by HSF-1 we generated a transcriptional fusion *daf-7::gfp* reporter containing 3.8 kb upstream regulatory sequences of *daf-7* gene (*pdaf-7::gfp*) and a mutated version of this reporter, in which 6 critical bases were removed from the putative HSF-1 binding site (p_{mut}*daf-7::gfp*). Both reporters were strongly expressed in the ASI neurons. However, while the wild-type reporter was

downregulated in *daf-11(-)* single mutants, expression of the mutant reporter was not significantly changed upon DAF-11 deficiency. In summary, we conclude that DAF-11/GC upregulates *daf-7* gene expression via inhibiting the activity of the HSF-1 transcription factor. Thus, HSF-1 acts as an upstream regulator of TGF-β signaling, and promotes dauer development at elevated temperatures via inhibiting *daf-7* transcription.

DAF-2/IR promotes *daf-7* expression via inhibition of HSF-1

- We showed, using a *daf-7::gfp* reporter (*ksIs2*), that absence of the insulin receptor DAF-2 leads to decreased *daf-7* expression in the ASI neurons of dauer larvae. However, *daf-2(e1370); hsf-1(sy441)* double mutant dauer larvae displayed wild-type levels of *daf-7* transcripts. A similar result was obtained in L1 stage larvae.
- We showed that overexpression of *hsf-1* enhances dauer larva formation in *daf-2(e1370)* mutant background at 20 and 23°C. Thus, DAF-2/IR promotes *daf-7* exp*ressi*on via inhibition of HSF-1, therefore HSF-1 interwines the systemic nutrient status sensing insulin/IGF-1 pathway and DAF-7/TGF-β signaling, thereby integrating distinct dauer formation inducing inputs.

HSF-1 also acts downstream of DAF-7/TGF-β to regulate aging and development

- We showed that lifespan extension in the *daf-7(-)* mutant background is suppressed by *hsf-1(sy441)*. This implies that *hsf-1* acts downstream of *daf-7* to regulate lifespan.
- We found that inactivation of HSF-1 increases, while its hyperactivation decreases the proportion of dauer larvae in *daf-7(-)* mutant background. We conclude that HSF-1 inhibits dauer development downstream of *daf-7*.

HSF-1 upregulates *daf-9/cytochrome P450*

- We have identified a conserved HSF-1 binding site in the upstream regulatory region of *daf-9*, using *in silico* methods.
- a *daf-9::gfp* reporter (*dhEx67*) containing the putative HSF-1 binding site is upregulated at 25°C in the hypodermis of L3 larvae, as compared with that obtained at 20°C. In *hsf-1(sy441)* mutant animals hypodermal upregulation of *daf-9::gfp* was not observed in L3 larvae. Thus we conclude that HSF-1 is required for temperature induced activation of *daf-9* transcription.
- *daf-9* expression is also upregulated when DAF-11/GC is depleted. We found that upregulation of *daf-9* in the hypodermis of *daf-11(-)* mutant L3 larvae was suppressed by *hsf-1(sy441)*, suggesting that HSF-1 is required for the activation of *daf-9* expression in *daf-11(-)* mutant background.

- In summary, *daf-9* may be another target of HSF-1, and HSF-1 modulates larval development at multiple points via inhibiting *daf-7* and also via activating *daf-9* expression.

***C. elegans* HSF-2 promotes reproductive development**

- *hsf-2* codes for a paralog of HSF-1 and contains an HSF-1 type DNA binding domain, but lacks any other domains characteristic for HSF family members.
- Penetrance of dauer constitutive phenotype of *daf-11(-)* and *unc-3(e151)* mutants defective in DAF-7/TGF-β signaling is enhanced in *hsf-2(tm4607)* null mutant animals. At the same time the penetrance of dauer constitutive phenotype of *unc-31(e169)* and *daf-2(e1370)* mutants defective in insulin/IGF-1 signaling is not affected by the *hsf-2(tm4607)* mutation. Since depletion of HSF-2 does not affect dauer constitutive phenotype of *daf-7(-)* mutant animals, we conclude that *hsf-2* acts parallel or upstream of *unc-31* to modulate insulin/IGF-1 signaling and larval development.

TGF-β signaling activates autophagy in *C. elegans*

- Using a *gfp::lgg-1* reporter for monitoring autophagic activity, we found that depletion of the TGF-β pathway component LON-1 promotes autophagy in hypodermal seam cells. *lon-1* expression is inhibited by DBL-1/TGF-β signaling, suggesting that DBL-1/TGF-β activates autophagy.

Conclusions

Most of the HSF-1 targets identified so far encode heat shock proteins protecting cells from protein-damaging agents [12]. Recently, it has been shown that HSF-1 also regulates the transcription of genes regulating non heat shock proteins [15, 16]. A few examples where HSF-1 transcriptionally represses non-heat shock proteins were also reported [17, 18]. In this study we showed that HSF-1 regulates the expression of two non-classical heat-shock genes, *daf-7*/*TGF*-β and *daf-9*/*cytochrome P450.*

We found that HSF-1 affects *C. elegans* larval development at multiple points. *daf-7* and *daf-9* are both components of the *C. elegans* TGF-β and steroid hormone receptor pathways, respectively. These pathways regulate the reproductive growth vs. dauer development decision. We showed that *daf-7* expression is repressed, while *daf-9* expression is upregulated by HSF-1, resulting in opposite effects on dauer formation. The overall impact of (dual) HSF-1 activity on this developmental choice (reproductive growth vs. dauer larva formation) is to promote dauer development in the wild type at high temperatures (over 25°C) with only a

moderate percentage. This way, HSF-1 regulates both a dauer-inhibiting (*daf-7*) and a dauer-inducing (*daf-9*) gene in order to allow the nematode populations to survive (some dauer larvae) and propagate (many reproductive adults) simultaneously under environmental stress, thereby maximizing the chance of the population to survive.

High temperature and population density, as well as starvation are environmental conditions that promote dauer development. According to our results, a complex regulatory interaction exists among the TGF-β, guanylate cyclase/cGMP and insulin/IGF-1 signaling systems, mediated by HSF-1, to affect development and lifespan in response to various environmental factors.

In this study we also showed that besides HSF-1 there is an additional heat shock transcription factor-like protein coding gene in the *C. elegans* genome. We initiated the genetic analysis of this gene called *hsf-2*. We found that *hsf-2* is also involved in the regulation of *C. elegans* dauer formation.

We also tested our hypothesis that the DBL-1/TGF-β pathway activates autophagy in hypodermal seam cells. Similarto autophagy [19, 20], TGF-β has a dual role in cancer: at early stages of carcinogenesis TGF-β has a tumor suppressor effect by inhibiting cell growth, while in later stages of cancer TGF-β promotes metastasis [21, 22]. These findings suggest that a regulatory relationship between TGF-β and autophagy exists and this plays a role in the formation and spreading of cancer.

Publications related to the doctoral thesis

1. Barna J, Princz A, Kosztelnik M, Takács-Vellai K, Vellai T. Heat shock factor-1 intertwines insulin/IGF-1, TGF-β and cGMP signaling to control development and aging. *BMC Developmental Biology.* 2012 Nov 1;12(1):32. [Epub ahead of print] IF: 2,78
2. Aladzsity I, Tóth ML, Sigmond T, Szabó E, Bicsák B, Barna J, Regos A, Orosz L, Kovács AL, Vellai T. Autophagy genes *unc-51* and *bec-1* are required for normal cell size in *Caenorhabditis elegans*. *Genetics*. 2007 Sep;177(1): 655-660. IF: 4.001 Independent citations: 13 Dependent citation: 5 Summary: 18

Other publications

3. Szabó E, Hargitai B, Regos A, Tihanyi B, **Barna J**, Borsos E, Takács-Vellai K, Vellai T. TRA-1/GLI controls the expression of the *Hox* gene *lin-39* during *C. elegans* vulval development. *Dev Biol*. 2009 Jun 15; 330 (2): 339-348. IF: 4.379 Független idéző: 3 Independent citations: 3 Dependent citation: 3 Summary: 6

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5. Tóth ML, Sigmond T, Borsos E, **Barna J**, Erdélyi P, Takács-Vellai K, Orosz L, Kovács AL, Csikós G, Sass M, Vellai T. Longevity pathways converge on autophagy genes to regulate life span in *Caenorhabditis elegans*. *Autophagy.* 2008 Apr 1; 4(3): 330-338. IF: 5.479 Independent citations: 100 Dependent citation: 8 Summary: 108

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